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Direct Analysis of Filter Paper Blood Specimens for Identification of Smith-Lemli-Opitz Syndrome Using Time-of-Flight Secondary Ion Mass Spectrometry

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In this study, time-of-flight secondary ion mass spectrometry was used to distinguish between blood of normal infants and that of individuals with Smith-Lemli-Opitz (SLO) syndrome. SLO syndrome results in an abnormally low concentration of blood cholesterol and an elevated concentration of 7-dehydrocholesterol. Blood was spotted on filter paper and analyzed directly with no extractions or separations. Results showed that using ratios of fragment ions for cholesterol/dehydrocholesterol, patients with SLO and normal individuals could be unambiguously distinguished. Unknown samples from 28 individuals were obtained and identified correctly. Am. J. Med. Genet. 68: 300–304, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: time-of-flight secondary ion mass spectrometry; Smith-Lemli-Opitz syndrome; blood; cholesterol; 7-dehydrocholesterol

INTRODUCTION

Neonatal screening has become standard practice in most industrialized nations, with virtually every newborn infant screened routinely for phenylketonuria (PKU) and congenital hypothyroidism. Both conditions result in severe mental retardation if not diagnosed neonatally and treated immediately. Many newborn screening programs have also added tests for additional conditions including sickle-cell anemia, galactosemia, maple syrup urine disease, homocystinuria, congenital adrenal hyperplasia, biotinidase deficiency, and cystic fibrosis. The supplemental newborn screening program in place in Pittsburgh is unique in that it offers screening for over 35 disorders using a variety of methods including acylcarnitine and amino acid profiling by tandem mass spectrometry for inborn errors of fatty acid, organic acid, and amino acid metabolism [Millington et al., 1990; Chace et al., 1993]. Screening is generally carried out on a capillary blood specimen collected by heel stick and spotted on a filter paper card, which in turn can be mailed to a central laboratory for testing.

We are also actively involved in a search for new analytical methods that will permit the detection of additional significant and potentially treatable conditions utilizing the filter paper blood specimen. Direct analysis on the filter paper specimen has a number of advantages, including the elimination of separation (chromatography) steps, reduction of introduced contamination, reduction of analysis time, and potentially reduced per-specimen cost. One technique that has shown promise for direct analysis is time-of-flight secondary ion mass spectrometry (TOF-SIMS) [Niehuis et al., 1987; Schweiters et al., 1991; Meyer et al., 1992]. TOF-SIMS offers both sensitivity and high-mass resolution, which enable unambiguous determination of the analyte; TOF-SIMS can be used for the analysis of samples which are complex matrices [Zimmerman et al., 1994]. The present study demonstrates the successful application of TOF-SIMS to the direct analysis of dried filter paper blood specimens and its use in the detection of Smith-Lemli-Opitz (SLO) syndrome [Smith et al., 1964].

Clinically, SLO syndrome is quite variable, but is generally characterized by microcephaly, growth retardation, midface dysplasia, syndactyly, polydactyly, cataracts, heart and kidney malformations, and mental retardation. Recently, a biochemical marker has been shown to be present in a large majority of SLO patients [Tint et al., 1994]. This defect results in a significant reduction in blood cholesterol and a marked increase in

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its precursor, 7-dehydrocholesterol. The frequency of SLO syndrome has been estimated to be 1 in 20,000 newborns [Opitz et al., 1994]. Until now there has been no screening test that could be routinely applied to the newborn filter paper specimen. Techniques such as routine biochemical assays and tandem mass spectrometry have not as yet been successful [Opitz and de la Cruz, 1994]. Currently, the preferred method of diagnosis involves capillary gas chromatography-mass spectrometry of plasma sterols following solvent extraction [Axelson, 1991].

MATERIALS AND METHODS

Analyses were performed using an Ion-Tof (Münster, Germany) TOF-SIMS III. This instrument uses a primary ion beam bombarding a surface with 10-keV argon ions to produce secondary organic ions, and has been described in detail elsewhere [Meyer et al., 1992]. The primary ion beam is pulsed and has a repetition rate of 5 kHz. The primary ion current used for obtaining spectra is about 0.5 pA with a pulse length of 800 ps. Charge compensation was accomplished using a 10-eV electron beam pulsed out of phase with the extraction field and primary ion beam. Spectra were obtained using the ion counting mode with a time-to-digital (TDC) resolution of 472 ps and total time range of 160 msec. Spectra were accumulated for 100 sec from an area of approximately 100 mm^2.

The filter paper whole-blood plasma specimens were supplied by Magee-Womens Hospital and Neo Gen Screening, Inc. (Pittsburgh, PA), the Kennedy Krieger Institute (Baltimore, MD), and the New England Regional Newborn Screening Program (Boston, MA). The filter paper samples (3.2-mm discs) were introduced directly into the transfer chamber and pumped down to a pressure of about 5×10^{-6} mbar before introduction into the main chamber. The 7-dehydrocholesterol and cholesterol standards were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Figure 1a shows a portion of the TOF-SIMS spectrum for the mass range of 360–390 Da from blood on filter paper from an infant with normal blood chemistry. The spectrum was obtained directly from the filter paper with no sample pretreatment. The main peaks of interest for cholesterol are $[M - H]^+$ (385.35 Da) and $[M - OH]^+$ (369.35 Da). The mass accuracy from spectrum to spectrum was about ±0.03 Da, with an approximate mass resolution ($m/\Delta m$) (measured at half-height) of 3,000. The resolution and intensity for cholesterol were somewhat better when using a plasma sample collected on filter paper, as shown in Figure 1b. The spectrum of a blank filter paper specimen shows virtually no peaks in this region. Figure 1c shows a cholesterol standard deposited from solution on filter paper. The pattern obtained from the cholesterol standard closely matches the pattern present in the normal whole-blood and plasma specimens.

Figure 2a shows the same portion of a TOF-SIMS spectrum from a filter paper blood specimen from a patient with SLO syndrome. A large peak was present corresponding to $[M - H_2O - H]^+$ (365.31 Da) in the 7-dehydrocholesterol standard, as well as a small cluster of peaks due to $[M - H]^+$ (383.32 Da), $[M]^+$ (384.33 Da), and $[M + H]^+$ (385.34 Da). The intensity of the peak corresponding to $[M - OH]^+$ (369.34 Da) for cholesterol was significantly smaller than that seen in a normal speci-

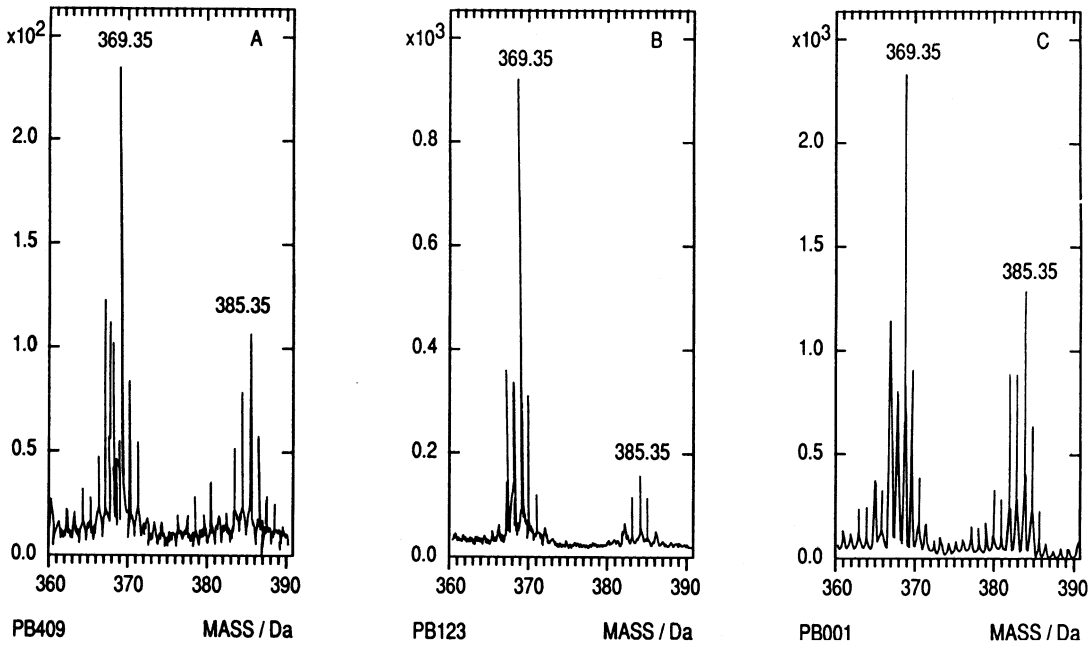

Fig. 1. TOF-SIMS spectra. **A:** Filter paper whole-blood specimen from normal individual. **B:** Filter paper plasma specimen from normal individual. **C:** Cholesterol standard on filter paper.

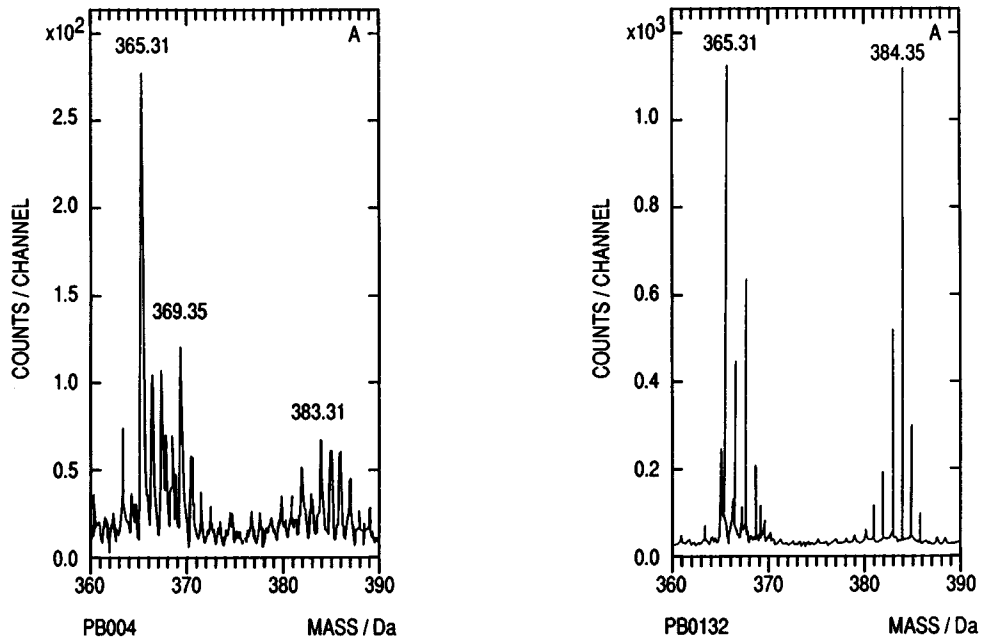

Fig. 2. TOF-SIMS spectra of **A:** Filter paper whole-blood specimen from SLO patient. **B:** 7-dehydrocholesterol standard on filter paper.

men. Intuitively, it is expected that $[M - OH]^+$ (367.35 Da) should be observed for 7-dehydrocholesterol; however, an additional loss of H_2 is not an uncommon process using an ion beam in SIMS [Zimmerman et al., 1993]. Figure 2b shows a TOF-SIMS spectrum of the 7-dehydrocholesterol standard. The $[M]^+$ (384.33 Da) peak is larger than the $[M - H]^+$ (383.32 Da) peak in the standard, in contrast to the SLO blood sample. This is most likely due to a combination of the instability of the 7-dehydrocholesterol, the impurity of the 7-dehydrocholesterol standard, and the presence of other isomeric dehydrocholesterols in plasma from SLO patients.

Next, 28 blinded unknown specimens were obtained from normal individuals and from individuals with SLO syndrome. These were analyzed using TOF-SIMS, and each sample was identified correctly with no false-positives or false-negatives. Figure 3a,b shows two spectra from the unknown set. Table I summarizes the

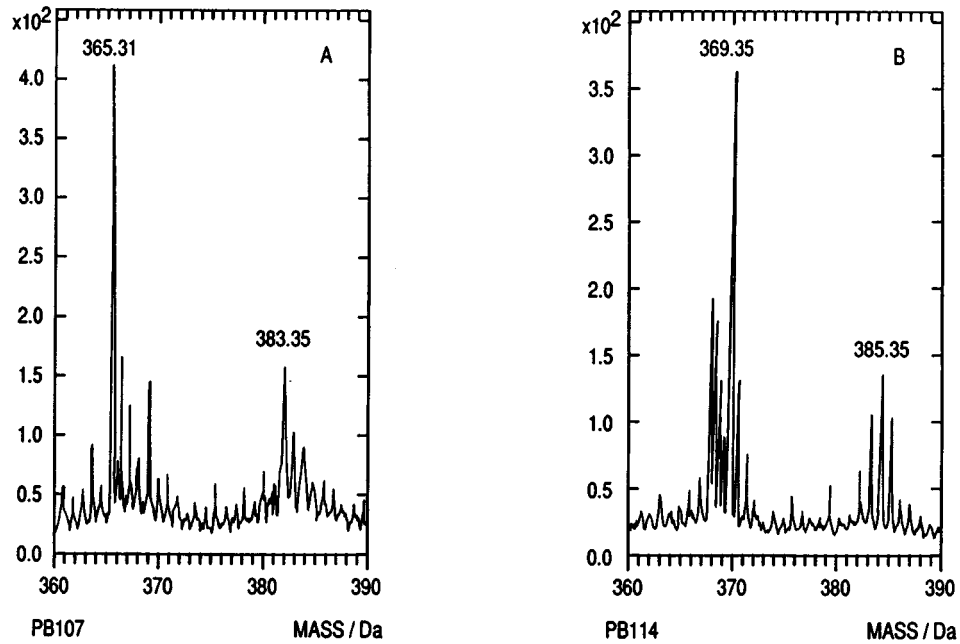

Fig. 3. TOF-SIMS spectra from unknown sample set. **A:** SLO patient. **B:** Normal individual.

TABLE I. Summary of Specimens Measured*

Unknown	Intensity ratio 369.3/365.3	Patient age	Diagnosis	Specimen date	Storage[a]
1	10.80 ± 0.40	30 years	SLO parent	04/04/94	−20°C
2	13.10 ± 0.20	3 years	SLO phenocopy	05/12/94	−20°C
3	0.23 ± 0.01	21 years	SLO syndrome	05/11/94	−20°C
4	11.80 ± 0.80	31 years	SLO parent	04/05/94	−20°C
5	1.06 ± 0.05	1 day	SLO syndrome	11/30/94	R.T.
6	12.50 ± 0.80	12 years	SLO phenocopy	11/10/93	R.T.
7	11.20 ± 0.50	20 mos	Multiple congenital anomalies	09/27/93	R.T.
8	1.26 ± 0.05	7 years	SLO syndrome	01/31/94	R.T.
9	1.60 ± 0.11	12 years	SLO syndrome	01/03/94	R.T.
10	12.10 ± 0.60	8 years	SLO phenocopy	10/04/93	R.T.
11	18.00 ± 1.40	—	Normal plasma	—	−20°C
12	18.20 ± 1.40	—	Normal plasma	—	−20°C
13	10.80 ± 0.10	—	Normal plasma	—	−20°C
14	14.20 ± 0.50	—	Normal plasma	—	−20°C
15	0.38 ± 0.06	2.5 years	SLO syndrome	01/28/94	R.T.
16	12.90 ± 0.40	2 days	Normal newborn	—	R.T.
17	11.20 ± 0.70	2 days	Normal newborn	—	R.T.
18	14.10 ± 1.10	2 days	Normal newborn	—	R.T.
19	15.30 ± 1.20	2 days	Normal newborn	11/28/89	R.T.
20	1.60 ± 0.19	2 days	SLO syndrome	11/28/89	R.T.
21	15.00 ± 1.20	2 days	Normal newborn	11/28/89	R.T.
22	14.00 ± 0.70	2 days	Normal newborn	—	R.T.
23	3.40 ± 0.90	2 days	SLO syndrome	—	R.T.
24	17.10 ± 1.50	2 days	Normal newborn	—	R.T.
25	0.29 ± 0.02	—	SLO syndrome	—	−20°C
26	0.31 ± 0.04	—	SLO syndrome	—	−20°C
27	1.80 ± 0.04	4 days	SLO syndrome	01/20/92	R.T.
28	1.64 ± 0.11	2 days	SLO syndrome	11/27/93	R.T.

*All measured on dried filter paper whole-blood specimens, except for 11–14 which were plasma samples all spotted on filter paper. Dashes, unknown
[a] R.T., room temperature.

ratio of the 369.35-Da peak (cholesterol) to the 365.31-Da peak (dehydrocholesterol) for the 28 samples analyzed. The ratios that are ≥10.0 are from individuals not affected with SLO syndrome. Samples 1, 2, 4, 6, 7, 10–14, 16–19, 21, 22, and 24 were from normal individuals. Samples 3, 15, 25, and 26 were from patients with typical severe SLO syndrome. Patients ranged in age from newborn to 21 years old. These specimens were either stored at −20°C or were freshly collected. Samples 5, 8, 9, 20, 23, 27, and 28 were from newborns with SLO syndrome, and were either stored at room temperature for up to 5 years. Additionally, Table I gives the patients' age, time of sample collection, and mode of sample storage. It therefore appears that specimens from the clinically and biochemically abnormal cases have 369.3/365.3 intensity ratios <0.5, provided they are tested fresh or stored frozen. If stored for some time at room temperature, the ratios are 1.06–3.40, which are still clearly abnormal. This is consistent with the suspected instability of 7-dehydrocholesterol when stored at room temperature. Despite the sample storage conditions, clear visual distinctions could be made between the two spectra using the $[M - H_2O - H]^+$ (365.31 Da) peak, which is present with high intensity in SLO patients, and nearly absent in normal individuals.

DISCUSSION

The screening test described here for the biochemical defect in SLO syndrome is simple and reliable. It can detect an abnormal 369.3/365.3 intensity ratio in dried filter paper blood specimens collected from newborns as well as from older patients. It meets most of the criteria for new screening tests set forth by the World Health Organization [1968] and the National Academy of Sciences (USA) [1975]. Its repeatability and accuracy have been demonstrated. The sensitivity and specificity of the assay appear to be good, but remain unknown pending appropriate pilot programs or field trials. While the initial cost of the equipment is high, the per-specimen cost is relatively low and the assay can be automated. From our preliminary studies it also appears that the 369.3/365.3 intensity ratio is sufficiently stable to permit reliable newborn screening.

Also, in order for routine newborn screening for an inherited metabolic condition to be acceptable, the seriousness and incidence of the disorder must be sufficiently high; the prognostic diagnosis of individuals identified by screening must be accurate; and there must be some benefit to the patient, his family, and society. With regard to SLO syndrome, the seriousness of the condition and its relatively high incidence have been well-documented. The prognostic diagnosis of individuals detected by screening remains to be determined and must also await the outcome of pilot programs and field trials. Finally, there is some optimism that some form of dietary therapy may be effective in some patients with SLO syndrome. If such therapy is to be effective, it is likely that early diagnosis through newborn screening will be essential.

This study clearly indicates that TOF-SIMS has the potential to be a viable technique for screening for SLO syndrome in newborns. There is a clear difference in spectra obtained from blood spots from normal individuals and from those afflicted with the disorder. The appropriate TOF-SIMS spectra can be obtained directly from blood specimens spotted on filter paper cards without additional sample preparation. It should also be possible to quantitate cholesterol and 7-dehydrocholesterol directly on the filter paper, since previous work involving quantitation on paper has yielded reasonable success [Zimmerman et al., 1994b]. Additionally, TOF-SIMS continues to be investigated as a technique for the routine screening of newborns for other inherited metabolic disorders.

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